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Environmental Effects of Dredging Technical Notes

Initial Comparisons of Six Assays for the Assessment of Sediment Genotoxicity

Purpose

This technical note reports and compares initial results of six genotoxicity bioassays applied to dredged sediments and describes progress toward development of a testing protocol to aid in regulatory decisionmaking when genotoxic chemicals are an issue of concern.

Background

The Long-term Effects of Dredging Operations Program work unit "Genotoxicity of Contaminated Dredged Material" was initiated in fiscal year 1990 to develop methods for assessing the genotoxic potential of dredged sediments. The impetus driving this new research and development effort was specific regulatory language in section 103 of the Ocean Dumping Act (Marine Protection, Research, and Sanctuaries Act (MPRSA) of 1972) prohibiting the open-water discharge of "mutagenic, carcinogenic, or teratogenic" substances in other than trace amounts, and language less specific but of similar intent in section 404 of the Clean Water Act (CWA).

At the time the genotoxicity work unit was begun, few tests of this kind had been applied to dredged sediments, and none were well understood or generally accepted. It was apparent that with a statutory mandate on the books, the unavailability of technically sound methods for addressing genotoxic potential in sediments constituted a regulatory time bomb.

At a workshop held at the U.S. Army Engineer Waterways Experiment Station (WES) (Reilly and others 1990), participants evaluated the state of the art in genetic and developmental aquatic toxicology and agreed upon an approach that would lead to interpretable and meaningful genotoxicity testing methods

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for dredged sediments. Since that time, efforts have been made at WES to adapt and test a suite of methods that show the highest potential for sediment genotoxicity testing in terms of ease of use, interpretability, reliability, and capability for application within the tiered testing framework established in the testing manual for section 103 of the MPRSA, the "Green Book" (USEPA/USACE 1991) and the draft Inland Testing Manual (USEPA/USACE 1994) for section 404 of the CWA.

Additional Information

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Introduction

Genotoxicity in the strictest sense refers to damage caused by reactions of foreign chemicals with nucleic acids (DNA and RNA) of cells (Jarvis, Reilly, and Lutz 1993). The results may be manifested as mutations, cancers, or developmental abnormalities if the damage is not repaired by the cellular defenses of the organism. Many environmental contaminants are genotoxic, and aquatic organisms such as polychaetes and particularly fishes are highly susceptible to genotoxicities. Aquatic crustaceans and molluscs are much less susceptible, but are not immune to these kinds of effects.

Not all cancers and developmental abnormalities are caused by genetic damage. For the purposes of testing dredged materials, the distinction is important only in test methods development, not in their application. Consequently, the suite of tests being developed at WES for detecting contaminants that are "mutagenic, carcinogenic, and teratogenic" in dredged sediments will include both biochemical endpoints, which are most appropriate for detecting damage to genetic material, and morphological endpoints, which are observable in early life stages of whole organisms. In addition, long-term testing methods where fish are exposed to cancer-causing chemicals and observed for the development of neoplasms and other cancerous lesions are under development at the U.S. Army Biomedical Research and Development Laboratory, Fort Detrick, MD. Although these latter methods may not be suitable for routine testing of dredged sediments, they provide a means for assessing the predictive capability of sediment genotoxicity bioassays intended for use in a regulatory framework.

Genotoxicity tests can be grouped into three categories: general indicators of genotoxic potential, biomarkers of exposure to genotoxic agents, and integrators

of genotoxic effects (Reilly and others 1990). General indicators of genotoxic potential can be applied to aqueous or organic extracts of sediments. These include bacterial tests of mutagenicity, tests for DNA damage in cell cultures exposed to the sediment extracts, tests for the induction of microsomal enzymes, and cytogenetic methods such as tests for micronuclei, anaphase aberrations, and sister chromatid exchange. The tests that are the subject of this technical note are general indicators of genotoxic potential. The second category, biomarkers of exposure, are tests applied to tissues of organisms exposed to genotoxic agents, as well as analyses for bile metabolites of specific compounds, and tests for the induction of metabolizing enzymes. The third category, integrators of genotoxic effects, includes effects on whole organisms, either aberrant morphologies in embryos and larvae or tumors and cancerous lesions in adults. Research on methods for the second and third categories has not yet been undertaken at WES.

Approach

Indicators

Six procedures representing three types of general indicators of genotoxic potential were selected for evaluation (Table 1). The three types of tests are complementary in terms of information obtained regarding genotoxic potential. The two examples of each type produce similar information. Differences among the types of tests are in the responsiveness to classes of genotoxic agents, sensitivity of the test, ease of performance, and potential for application in a routine testing framework.

Table 1. General Indicators of Genotoxic Potential Applied to Sediment Extracts

Type of Indicator	Test
Mutagenicity	(1) Ames Test (2) Mutatox[1]
DNA strand breaks	(1) Alkaline unwinding assay (2) Single cell gel assay
Enzyme induction	(1) H4IIE in vitro assay (2) P450 Reporter Gene System[1]

[1] Proprietary assay.

Sediments

Sediments with varying degrees of contamination were selected from the WES inventory for testing with the six bioassays described above. The sediments were soxhlet extracted according to EPA method 3540 (USEPA 1986), cleaned up on silica gel columns (Warner 1976), and the resulting extracts were solvent-exchanged into DMSO for bioassay.

Comparison of Methods

Mutagenicity

A mutation is a change in the DNA of a cell capable of being passed on to the next cell generation (Klaassen and Eaton 1991). Not all mutations are detrimental to the cell or organism, and a few may be advantageous. Most, however, are either silent or dysfunctional, and some mutations are lethal, causing a critical cellular process to be compromised and the cell or organism dies. A mutation can also lead to altered gene expression, resulting in a variety of possible outcomes, including cancer, immune suppression, teratogenesis, or genetic disorders.

Ames Test. The Ames Test is the most widely used test for mutagenicity. It has been estimated that 80 to 90 percent of the chemicals showing mutagenicity in the Ames Test are carcinogenic in mammals (Maron and Ames 1983). This assay uses selected strains of the bacteria *Salmonella typhimurium*, mutated so that they can no longer synthesize histidine, a vital amino acid. The bacteria and the test material are incubated together and placed in agar that does not contain histidine. Bacteria that live under these conditions have undergone a mutation back to the "wild type" capable of manufacturing their own histidine. The formation of bacterial colonies on the agar indicates that the test material has mutagenic potential. Drawbacks to the test are the requirements for sterile technique, numerous quality control tests, and a relatively high degree of technical expertise in the performance of the test. A chief advantage is a well-developed methodology that includes numerous variations on the test capable of detecting many genotoxic modes of action. The Ames Test is in the public domain, making it widely available to potential users. Strains of the bacteria used in the test are available from Dr. Bruce Ames, University of California, Berkeley.

Mutatox. A proprietary mutagenicity assay was selected for comparison with the Ames Test. Mutatox (Johnson 1992) also uses bacteria, the luminescent *Vibrio fischeri*, which has been mutated so that it is no longer luminescent. Reversion of the bacteria after exposure to a mutagen restores luminescence, which is measured using a luminometer. The standard method used to perform the Mutatox assay is described in detail by the manufacturer (Microbics Corporation 1993). Advantages of the method include its simplicity in the quantitation of the response as the amount of light produced, and the fact that it is a relatively simple and rapid technique. Disadvantages include a much higher start-up cost than is required for the Ames Test and a limited database for the interpretation of results.

Comparison of Results. Mutagenicity test results are shown in Table 2. The Ames assay was performed using 10 sediments from different locations. The soxhlet extracts were split into two aliquots, one of which was cleaned using silica gel (clean extract) and the other was not cleaned (crude extract), resulting in 20 extracts. Silica gel cleanup removes biogenic polar compounds

Table 2. Mutagenicity Test Results of 20 Sediment Extracts Using Two Tester Strains of the Ames Test and Mutatox

Sediment	Ames Assay[1]		Mutatox
	TA100	TA98	
Sandy Hook, Clean	-	-	-
Sandy Hook, Crude	-	-	-
Gowanus Creek, Clean	+	+	+
Gowanus Creek, Crude	-	+	+
Arthur Kill, Clean	+	+	+
Arthur Kill, Crude	-	+	+
Red Hook, Clean	+	+	+
Red Hook, Crude	-	+	Mixed[2]
Chicago CDF,[3] Clean	+	+	+
Chicago CDF, Crude	+	+	+
Hamlet City, Clean	-	+	+
Hamlet City, Crude	-	-	-
Oakland Reference, Clean	+	+	+
Oakland Reference, Crude	+	-	+
Oakland Inner, Clean	+	+	-
Oakland Inner, Crude	-	+	+
Oakland Outer, Clean	+	-	Mixed
Oakland Outer, Crude	-	+	+
Oakland Hot, Clean	-	+	-
Oakland Hot, Crude	-	+	+

[1] Positive (+) or negative (-) for mutagenicity.
[2] Positive and negative results obtained from multiple tests.
[3] Confined disposal facility.

that are residuals of the extraction process. The same 20 sediment extracts were sent to Microbics Corporation for Mutatox testing. Table 2 shows that comparable results, both positive and negative, were obtained with most of the 20 sediment extracts using two Ames Test bacteria tester strains (TA98 and TA100) with metabolic activation and the Mutatox system. The TA98 test strain responds specifically to frameshift mutations while TA100 responds to base pair substitutions (Zeiger 1985). Mutatox can detect both types of mutations in addition to compounds that intercalate with DNA (Ulitzer, Weiser, and Yannai 1980, 1981).

Based on sediment analytical data not reported in this technical note, both assays detected mutagenicity in suspect sediments and did not indicate mutagenicity in nonsuspect sediments. For example, Gowanus Creek and Arthur Kill, both of which have relatively high polycyclic aromatic hydrocarbon (PAH)

content in the sediments, were identified as mutagenic by both assays. Conversely, Sandy Hook sediments, which have low concentrations of anthropogenic chemical contaminants, were identified as nonmutagenic by both assays. In two cases (Oakland Inner, Clean and Crude), Mutatox failed to detect mutagenicity identified by both tester strains in the Ames Test. In one other case (Oakland, Hot Clean), Mutatox failed to detect mutagenicity detected by one of the Ames Test strains (TA98) that responds specifically to frameshift mutation.

DNA Strand Breaks

Some genotoxic chemicals act by breaking one or both strands of the DNA molecule (Daniel, Haas, and Pyle 1985). Assays that detect DNA strand breaks generally measure the characteristic unwinding of DNA from the double-stranded form to the single-stranded form that occurs when it is in an alkaline environment. The rate of DNA unwinding is directly proportional to the number of strand breaks in the DNA.

Alkaline Unwinding Assay. In the alkaline unwinding assay described by Daniel, Haas, and Pyle (1985), cells (H4IIE rat hepatoma cells for the work described herein) are incubated with a test compound for 6 hr in culture dishes and then subjected to the assay. Alkaline unwinding is measured fluorometrically using Hoechst 33258 dye, which binds specifically to double-stranded DNA and is expressed as an F value, the fraction of double-stranded DNA remaining after 30 min of unwinding. An advantage of the alkaline unwinding assay is that it is technically simpler to perform than is the single cell gel assay. However, the single cell gel assay appears to have greater sensitivity. Both assays detect the same type of damage.

Single Cell Gel Assay. The single cell gel assay developed by Singh and others (1988) utilizes the alkaline unwinding principle, but H4IIE cells are unwound for 20 min after being embedded in an electrophoresis gel. Double- and single-stranded DNA are separated by electrophoresis and visualized using a fluorescence microscope after staining with ethidium bromide. Start-up cost due to the equipment required is approximately 15-fold greater than required for the alkaline unwinding assay.

Comparison of Results. Testing of the cleaned extracts using the Alkaline Unwinding Assay is ongoing. However, preliminary results for three sediment extracts are shown in Table 3.

Since a lower value indicates DNA damage, the results appear to suggest that Hamlet City and Sandy Hook sediment extracts are genotoxic. However, these data are only preliminary, since too few tests have been performed for adequate evaluation.

Single cell gel assays using cleaned extracts are also ongoing, with preliminary data shown in Table 4 for two sediment extracts analyzed to date: Chicago confined disposal facility (CDF), a contaminated sediment, and Hamlet City, a suspect sediment. These preliminary data indicate that the Chicago

Table 3. Preliminary Results of the Alkaline Unwinding Assay for DNA Strand Breaks Using a Cultured Cell Line Exposed to Three Sediment Extracts			
	Sediment Extract Dilution, percent		
Sediment	10[1]	50	100
Chicago CDF	105.6[2]	112.7	94.4
Hamlet City	96.2	76.9	71.1
Sandy Hook	92.3	81.5	87.7

[1] Extracts solvent-exchanged into DMSO.
[2] Values indicate fraction of undamaged DNA, expressed as percent of control.

Table 4. Preliminary Results of the Single Cell Gel Assay for DNA Strand Breaks Using a Cultured Cell Line Exposed to Two Sediment Extracts			
	Sediment Extract Dilution, percent		
Sediment	10[1]	50	100
Chicago CDF	400[2]	262	154
Hamlet City	167	300	256

[1] Extracts solvent-exchanged into DMSO.
[2] Values indicate number of cells damaged per 100 cells, expressed as percent of control.

CDF extract damaged DNA at all concentrations, as evidenced by more strand breaks in all treatments than control, and also produced cytotoxicity at the higher two concentrations, indicated by the decreasing number of DNA strand breaks with increasing extract exposure concentration. The Hamlet City extract was also apparently genotoxic, demonstrating a dose-responsive increase in DNA strand breaks above control levels. Both the single cell gel and the alkaline unwinding assays are undergoing further optimization and evaluation.

Enzyme Induction

Induction (stimulated synthesis) of detoxifying enzymes occurs in metabolically active cells of mammals, birds, and fish exposed to certain classes of chemicals. Cytochrome P-4501A1 (CYP1A1)-dependent monooxygenases are a class of enzymes that are induced by exposure to specific organic chemicals including the PAHs, coplanar polychlorinated biphenyls, dioxins, and furans. These are among the most commonly encountered dredged sediment contaminants, and include carcinogens, procarcinogens, and promoters of carcinogenicity. The induction of specific monooxygenases caused by these compounds can be measured quantitatively and used as biomarkers of genotoxicant exposure in eggs or in whole organisms (Tillet, Giesy, and Ankley 1991) or as general indicators of genotoxic potential in cultured cell lines exposed to sediment extracts.

H4IIE in vitro Assay. The H4IIE in vitro assay uses a rat hepatoma cell line incubated with the test compound and allows time for enzyme induction to occur. Ethoxyresorufin-O-deethylase (EROD) activity is then measured fluorometrically as

a sensitive indicator of enzyme induction. As used in the preliminary experiments reported in this technical note, the test has a lower limit of detection of approximately 10^{-11} g (10 picogram) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD). The test can be standardized against 2,3,7,8-TCDD and the results expressed as toxic equivalents (McFarland, Clarke, and Ferguson 1993). The cell line is public domain and readily available.

P450 Reporter Gene System. The P450 Reporter Gene System (P450 RGS) is based on a genetically engineered cell line, and the assay is proprietary. A human hepatoma cell line (HepG2) is used as the basis of the assay. The HepG2 was modified by insertion of the gene for the firefly luciferase enzyme downstream from the CYP1A1 gene. Activation of the CYP1A1 gene results in the expression of luciferase, which is easily measured using a luminometer (Anderson and others 1993). Advantages of the P450 RGS include a shorter time requirement for the response and greater simplicity in instrumentation needs. H4IIE and P450 RGS measure responses to the same chemicals, but sensitivities appear to differ somewhat. P450 RGS appears to be less sensitive to PAH compounds than is H4IIE, and more sensitive to dioxin-like compounds.

Comparison of Results. Table 5 contains preliminary enzyme induction results for six sediment extracts tested using the H4IIE in vitro rat hepatoma bioassay and P450 RGS. An additional sample preparation step that selectively removes PAHs from extracts, sulfuric acid silica gel (SASG) reactive cleanup, was employed for these tests in addition to testing the silica gel-cleaned extracts. SASG reactive cleanup allows differentiation of the presence of PAHs from other contaminants. The results are expressed as average fold induction, which is calculated as the enzyme activity recorded at the end of the induction period divided by the enzyme activity in cells exposed only to the solvent blank. The P450 RGS average fold induction was measured after 16 hr of exposure and the H4IIE after 24 hr. The differences in the results show that 24 hr is clearly insufficient

Table 5. Comparison of Two Enzyme Induction Assays Performed on Six Sediment Extracts

	P450 RGS		H4IIE in vitro Assay	
	Average Fold Induction		Average Fold Induction	
Sediment	SASG-Treated	CleanedExtract	SASG-Treated	Cleaned Extract
Sandy Hook	4	6	1.14	0
Oakland Hot	51	61	1.73	1.92
Hamlet City	41	81	1.45	1.63
Passaic River	85	84	2.76	1.84
Newark Bay	102	107	2.50	3.01
Chicago CDF	110	113	2.88	3.10
TCDD standard	NA[1]	142[2]	NA	1.86[3]

[1] Not applicable.
[2] 6-picogram exposure.
[3] 100-picogram exposure.

for the H4IIE. Typically, the H4IIE cells are given 72 hr for induction to occur before measurements are made, and this time difference points to a clear advantage of the P450 RGS.

The sediment extracts were ranked in the same order of genotoxicity by both bioassays, with Chicago CDF being the most genotoxic and Sandy Hook being the least. The SASG cleanup step altered enzyme induction with both assays, generally decreasing genotoxicity, as would be expected by the removal of PAHs.

From these results it appears that the P450 RGS system is more sensitive than the H4IIE bioassay. However, the H4IIE bioassay was performed using large culture dishes while P450 RGS used a microtitre method. Dr. Donald Tillet reported the H4IIE bioassay to be more sensitive when using a microtitre method (personal communication).

Conclusions and Future Directions

Each of the six bioassays demonstrated genotoxic responses with suspect sediment extracts. Results from the Mutatox and the Ames Test correlated very well, as did results from the P450 RGS and the H4IIE bioassay. The Ames Test is a more established mutagenicity test than is Mutatox, although it is much more technically difficult to perform than Mutatox. However, the start-up cost of Mutatox is much greater than the Ames Test ($23,000 versus ~$3000), although the cost per assay is far greater for Ames than for Mutatox ($1,000-$3,000 versus $100). A suggested approach for using these assays would be to use Mutatox for primary screening of sediments with the Ames Test as a confirmation assay.

Data from the single cell gel and the alkaline unwinding assays are sparse and inconclusive, and much additional testing is required for satisfactory comparisons to be made. However, these assays are relatively simple and inexpensive to perform ($50 per sample) and hold promise as rapid initial screens of sediment genotoxicity.

The enzyme assays are highly sensitive and more specific than the mutagenicity and DNA strand break tests, and provide both confirmatory and complementary information. The P450 RGS is a proprietary assay owned by EMCON Marine Sciences/Columbia Aquatic Sciences and is performed for ~$200 per extracted sample. The H4IIE bioassay is under public domain and can be performed for about the same price. The technical requirements of the two enzyme assays are the same, both involving the use of sterile technique and cell culture. However, the rapidity of the RGS, possibly greater sensitivity, and simplicity of measurement conferred by the generation of luminescence rather than fluorescence are all advantages over the H4IIE assay.

The results obtained thus far indicate both similarities and differences between pairs of tests requiring further delineation. Before final recommendations can be made, the testing suite must be validated against fish cancer and early life

stage developmental aberration models. The next stage of testing will involve determining the sensitivity and selectivity of the assays, and refining the techniques. The tests must also be performed using aqueous as well as other organic sediment extraction methods to determine whether the potential for genotoxicity detected using soxhlet extracts can be related to bioavailability.

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